Genipa americana and *Ageratina anisochroma*, two new hosts of *Candidatus Phytoplasma asteris* in Costa Rica

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Abstract

We report two new plant species hosts for *Candidatus Phytoplasma asteris*: *Genipa americana* (Rubiaceae) and *Ageratina anisochroma* (Asteraceae). Phytoplasma infections were detected by real-time loop-mediated isothermal amplification and nested PCR. Consensus sequences from both hosts share 99% identity to the 16SrI-B subgroup using BLAST; however, potential new subgroups are suggested due to unique RFLP patterns of the 16S rDNA F2nR2 fragment.

Keywords Nested PCR · Real-time LAMP · Jagua tree · Guaitil tree

Phytoplasmas are pleomorphic prokaryotes that lack a cell wall (class *Mollicutes*) and live in plant phloem tissue and insect vectors (mainly species of Cicadellidae). Phytoplasmas cause plant diseases worldwide and have a negative impact on crops of economic interest as well as natural ecosystems (Hogenhout et al. 2008; Weintraub and Beanland 2006).

Symptoms associated with phytoplasma infection (Bertaccini 2007) were noticed in two native plant species during field surveys conducted since 2010. A jagua tree (*Genipa americana* L., Rubiaceae) exhibiting branches with witches'-broom, little leaves and yellowing (Fig. 1a) at Orotina region (9°53'31.63" N, 84°38'05.74" W, 91 masl) in the Pacific lowlands of Costa Rica (Puntarenas province). No flowers or fruits were observed on branches showing the mentioned symptoms during several visits to the area from 2010 to 2016. The symptomatic tree and two asymptomatic trees in the area were sampled (total of three *G. americana* samples). *Genipa americana* is a neotropical tree species native of wet or moist areas from southern Mexico to the north of Argentina. It is commonly known by different names in Costa Rica: jagua, guaitil or genipa. *G. americana* is used as a traditional treatment for anemia, icterus, asthma, liver and spleen

diseases (Conceição et al. 2011). Pharmacological trials showed antibacterial, antitumoral, anti-inflammatory and antioxidant activities in the fruit extracts (Omena et al. 2012). Additionally, the oxidized juice of the edible fruit has been commonly employed by Central and South American indigenous people to paint their faces and bodies, and to dye textiles.

The second native plant species that we noticed displaying phytoplasma-like symptoms was *Ageratina anisochroma* (Klatt) R.M. King & H. Robinson (Asteraceae). During a field trip (2017) to different locations at Bellavista Mountain, few patches of *A. anisochroma* plants showing axillary proliferation, little leaves and dwarfing were observed (Fig. 1b). Six symptomatic plants (per locality) were collected at La Cangreja (9°47'52.05" N, 83°57'55.07" W, 1889 masl, Cartago province) and at La Sierra (9°44'39.59" N, 83°58'36.05" W, 2024 masl, San Jose province), additionally two asymptomatic plants were also collected in each location.

Ageratina anisochroma (formerly *Eupatorium anisochromum* Klatt) is an endemic flowering shrub, up to 2 m tall known from Nicaragua, Costa Rica, and western Panama (King and Robinson 1970, 1972; Woodson et al. 1975), usually found above 1500 masl. This plant is a frequent pioneer species during secondary forest succession; therefore, the sampled patches were distributed along the sides of roads (sites of constant disturbance of the vegetation). Tamayo-Castillo et al. (1988) found germacranolides and several thymol derivatives in this species. This kind of compounds may have potential pharmacological activities, i.e. anti-inflammatory, antiseptic, antiviral, or antimycotic action.

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Fig. 1 **a** *Genipa americana* (jagua tree) with branches showing little leaves and yellowing symptoms observed near to Orotina town (Puntarenas province). **b** & **c** *Ageratina anisochroma* showing axillary proliferation and little leaves symptoms (**b**) and healthy plants (**c**) in a patch in La Sierra (San Jose province)

To determine if phytoplasmas were associated with symptoms observed in both wild species, DNA was extracted from each sample using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. As a first screening, the symptomatic *Genipa* tree and six of the symptomatic *Ageratina* plants, plus one asymptomatic sample of each plant species were evaluated by Real-time Loop-Mediated Isothermal Amplification (RT-LAMP) on a Genie II instrument, using the 2× Isothermal Master Mix (OptiGene), the three pairs of universal primers: UNIF3/ UNIB3, UNIFIP/ UNIBIP, and UNIFL/ UNIBL, and protocols described in Dickinson (2015). A negative control consisted of water as template, and DNA from *E. poeppigiana* infected with phytoplasmas of

16SrI group (Saborío-R et al. 2007) was used as a positive control. The DNA samples of symptomatic *Genipa* tree and *Ageratina*, as well as the 16SrI group positive control, produced a clear fluorescence signal curve, whereas non symptomatic samples and the water control showed a flat line (no fluorescence). RT-LAMP results indicated the presence of phytoplasmas; therefore, all the collected samples were tested by nested PCR using universal phytoplasma primers P1/P7 (Deng and Hiruki 1991; Duduk et al. 2013) followed by R16F2n/R16R2 (Gundersen and Lee 1996) in a total volume of 27 µL using 2× DreamTaq PCR Master Mix (Thermo Scientific), according to Villalobos et al. (2011). The expected amplification product (1200 bp) was detected only from symptomatic samples of

Genipa ($n = 1$) and *Ageratina* ($n = 12$), and the positive control. The *Genipa* tree sample was tested throughout 5 years (once per year) resulting in a consistent association of the symptomatic tissue with phytoplasmas detection through time. Likewise, consistent association for 16 different symptomatic plants was observed in the case of *A. anisochroma*. The PCR products obtained from the jagua tree and two *Ageratina* samples were directly sequenced in both directions (Macrogen Inc., Korea). Contig sequences were assembled using BioEdit (Hall 1999), and a BLASTn search was done (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phytoplasmas detected in both species share 99% identity to the 16SrI-B phytoplasma subgroup.

Semi-nested PCR reactions were run using primers P1/R16S-SR and P1A/R16S-SR (Lee et al. 2004). Amplifications were performed with a PCR Gradient Palm Cycler (Corbett Research Model CG1–96, Australia) in 25 μ L reactions containing 200 μ M of each of the four dNTPs, 0.4 μ M of each primer, 1.5 mM MgCl₂, 0.625 units of DreamTaq DNA polymerase (Thermo Fisher Scientific Inc. USA), and 1 μ L of diluted DNA. Amplicons (ca. 1.5 kb) were purified, cloned (TA system, Macrogen Inc., Korea) and sequenced in both directions for the symptomatic jagua tree, one *Ageratina* sample and *E. poeppigiana*. The resulting nucleotide sequences from three clones for each species were edited and one consensus sequence per species: *Ageratina anisochroma*, *Genipa americana* and *E. poeppigiana*, was obtained and deposited in GenBank (GB, Accession numbers: MH011345, MH011346 and MH011347, respectively).

A computer-simulated restriction fragment length polymorphism (RFLP) analysis was conducted on the 16S rDNA F2nR2 fragment of sequences from jagua and *Ageratina* using the iPhyClassifier program (Zhao et al. 2013). The phytoplasmas detected in those plant species had similarities of 98.3 and 97.9%, respectively, to the reference strain '*Candidatus* Phytoplasma asteris' (GB Acc. M30790) in accordance with previous BLAST results. However, the virtual RFLP patterns (from the 16S rDNA F2nR2 fragment) were different between them and from the reference patterns of all previously established 16Sr subgroups. Specifically, the restriction patterns were unique for *Genipa* and *Ageratina* phytoplasmas with enzymes *AluI* and *HaeIII*, and additionally for *Genipa* phytoplasma with *RsaI* (Fig. 2). The most similar pattern to *Ageratina*'s phytoplasma was the 16SrI-B subgroup pattern: Onion yellows phytoplasma (GB Acc. AP006628), with a similarity coefficient of 0.92. Meanwhile, *Genipa*'s phytoplasma showed a 0.82 similarity coefficient to the nearest reference pattern, corresponding to subgroup

16SrI-X: Papaya bunchy top phytoplasma (GB Acc. JF781308). Based on the consistent association of symptoms with the detection of phytoplasmas for *Genipa* and *Ageratina* samples and results (sequences and RFLPs) indicating two different phytoplasmas subgroups belonging to '*Ca. Phytoplasma asteris*', one associated with each plant species, we proposed the names *Ageratina* little leaf phytoplasma and *Genipa* yellowing phytoplasma for the corresponding related phytoplasma strains associated with the described symptoms.

Phylogenetic analyses were performed using the 16S rRNA region from a total of 23 sequences: three consensus sequences from Costa Rican samples and 19 reference strains (16SrI subgroups and '*Ca. Phytoplasma* spp.' retrieved from Gen Bank); and one *Acholeplasma palmae* as outgroup. Sequences were aligned with MUSCLE (Edgar 2004) algorithm in MEGA7 (Kumar et al. 2016). The alignment was trimmed to a consistent length of 1260 positions per sequence (nucleotides with gaps). A phylogenetic analysis was conducted with MrBayers v.3.2.6 (Ronquist et al. 2012) using a mixed substitution model (Iset = mixed) and nucleotide gamma-shaped rate variation across sites with a proportion of invariable sites (Iset rate = invgamma) for 30 million generations. The resulting dendrogram (Fig. 3) showed that *Ageratina* little leaf phytoplasma and *Genipa* yellowing phytoplasma clustered together with those of '*Ca. Phytoplasma asteris*' without associating to a specific subgroup. However, our positive control, *Erythrina* little leaf phytoplasma, associated with subgroup 16SrI-AG (GB Acc. AY249247). This phylogenetic tree indicated that phytoplasmas infecting *G. americana* and *A. anisochroma* belong to '*Ca. Phytoplasma asteris*' group but are different strains between them.

After 8 years of our first observation of the *Genipa* symptomatic tree, no more symptomatic trees were observed in the area. This observation suggests that this species tree may be an example of an incidental or dead-end host plant for this phytoplasma (Alma et al. 2000; Weintraub and Beanland 2006). Conversely, a putative different epidemiological scenario seems to occur with *Ageratina*: a patch with a proportion of symptomatic plants was found at two locations during 2017, and an additional patch near to La Sierra was found recently (February 2018). No symptomatic *A. anisochroma* plants were noticed in previous surveys to the Bellavista Mountain area. A gross estimation of symptomatic plants incidence per patch was near 4%; therefore suggesting a possible new plant host-phytoplasma interaction. Lee et al. (1998) speculated

Fig. 2 Virtual RFLP's to F2nR2 fragment of jagua's and *Ageratina*'s phytoplasmas showing different pattern between them as well as patterns found to all strains to *AluI* and *HaeIII* jagua's phytoplasma also showed a distinct virtual pattern with *RsaI*. Virtual RFLPs obtained in *iPhyClassifier* (<http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) and edited using *Paint.net* software

how distinct phytoplasma strains evolved. Those authors proposed that occasionally a polyphagous insect vector may feed on a non-host plant for the insect species. If the non-host plant is susceptible to the phytoplasma strain carried by the insect a new infection is established. Afterwards, there is opportunity for an insect species that regularly feeds on the newly infected plant to acquire and transmit the phytoplasma. In this way, other individuals of the new host species or alternative species may become infected.

The record of natural host plants for '*Ca. Phytoplasma asteris*' worldwide increases each year. Its occurrence in Costa Rica was already known (Saborio-R et al. 2007), herein we report two new wild hosts:

Genipa americana and *Ageratina anisochroma*. Both plant species were infected with different and potentially new 16SrI subgroups as suggested by comparison of 16S rDNA sequences and virtual RFLP's. It is of scientific and economic interest to register the establishment of phytoplasmas in new hosts in order to understand their ecology. Phytoplasmas spreading to new hosts and their diversification (Lee et al. 1998) may affect the survival of infected wild plants and the yield and/or economic value of cultivated plant species. To our knowledge, this is the first report of infection of *Genipa americana* and *Ageratina anisochroma* by '*Ca. Phytoplasma asteris*' or any other phytoplasma strain worldwide.

Fig. 3 Phylogenetic tree constructed using MrBayers from the 16S rRNA gene sequences of 22 phytoplasmas sequences (three Costa Rican and 19 reference strains) and *Acholeplasma palmae* as outgroup. GenBank accession number to all sequences used are showed in the dendrogram

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